ISSN: 2573-962X

Research Article

Journal of Pharmaceutical Research

Phytochemical Screening, Acute Toxicity and Anti-Rabies Activities of Extracts of Selected Ethiopian Traditional Medicinal Plants

Yeweynshet Tesera[1,2*], Asnake Desalegn[2], Ashenif Tadele[1], Abebe Mengesha[1], Birhanu Hurisa[1], Jemal Mohammed[1], Demise Mulugeta[1], Sintayehu Ashenafi[1], Anberbir Alemu[1], Sileshi Degu[1] and Ayele Bizuneh[1]

[1]*Ethiopian Public Health Institute, Addis Ababa, Ethiopia*

[2]*Addis Ababa University, College of Natural and Computational Sciences, Department of Microbial, Cellular and Molecular Biology, Addis Ababa, Ethiopia. P.O. Box.1176, Addis Ababa, Ethiopia*

[*]Corresponding author
Yeweynshet Tesera, Ethiopian Public Health Institute, Addis Ababa, Ethiopia and Addis Ababa University, College of Natural and Computational Sciences, Department of Microbial, Cellular and Molecular Biology, Addis Ababa, Ethiopia. P.O. Box.1176, Addis Ababa, Ethiopia.

Submitted: 15 Feb 2022; **Accepted**: 26 Feb 2022; **Published**: 15 Mar 2022

Citation: *Yeweynshet Tesera, Asnake Desalegn, Ashenif Tadele, Abebe Mengesha, Birhanu Hurisa, et al. (2022). Phytochemical Screening, Acute Toxicity and Anti-Rabies Activities of Extracts of Selected Ethiopian Traditional Medicinal Plants. J Pharmaceut Res, 7(1), 150-158.*

Abstract

Background: *Croton macrostachyus, Justicia schimperiana, and Ricinus communis have been widely used for the traditional treatment of rabies in Ethiopia. The objective of this study was to investigate the phytochemical constituents, acute toxicity, and antirabies activity of crude extracts of the leaves of Justicia schimperiana and Ricinus communis and the stem bark of Croton macrostachyus.*

Methods: *In studying the presence of phytochemicals within each plant extract, standard procedures were used. The Organization for Economic Corporation and Development (OECD) Guideline No.423 was used for the determination of acute toxicity and antirabies activities of extracts towards Swiss albino mice. Different concentrations of extracts were tested for their cytotoxic effect on Vero cells through 3-(4, 5-Dimethylthiazol-2-yl)-2, 5Diphenyltetrazolium Bromide (MTT) assay. The antirabies assay was carried out based on the minimal toxic concentration of extracts.*

Results: *The phytochemical constituents identified were alkaloids, flavonoids, phenols, steroids, tannins, and terpenoids. All the extracts were slightly toxic in the Swiss albino mice model but noncytotoxic in Vero cell lines. The antirabies assay result showed that all plant extracts had a moderate to good antirabies potential. The methanol extracts exhibited an appreciable antirabies activity compared to the other extracts under investigation.*

Conclusion: *The present study concluded that the studied plant extracts have possessed different phytochemicals and have shown appreciable antirabies activities. Hence these plants could be a potential source of the novel antirabies compound and also may be effective against other viruses.*

Keywords: Anti-Rabies, Pathogenicity, Phytochemicals, Traditional Medicinal Plants

Introduction

Rabies, a neglected viral zoonosis, is still a major public health problem all over the world which causes more than 50, 000 human deaths annually primarily in developing countries of Asia and Africa [1]. It is caused by a neurotropic, negative sense, non-segmented, single-stranded RNA virus that belongs to the Lyssavirus genus of the Rhabdoviridae family and Mononegavirale order [2]. It is a fatal disease in humans, and, to date, the only survivors of the disease have received rabies vaccine before the onset of illness [3]. Although effective and economical control measures (PEP) are available, rabies remains a neglected disease throughout most of the developing world and hence, more than 99% of all human deaths from rabies occur in these countries [4]. The endemicity of rabies in developing countries could be attributed to lack of risk communication, poor surveillance of rabies-related viruses and poor diagnostic capability, low vaccine coverage, and failure to immunize domestic dogs.

Rabies has been recognized as an important disease for many centuries in Ethiopia [5]. Dogs were the dominant species responsible

for the transmission of rabies virus to humans and livestock and it has been a common practice to provide post-exposure vaccines to humans bitten by dogs irrespective of their rabies status [6]. The continuing burden of the disease in Ethiopia has forced the scientific community to investigate less toxic, affordable protective biologicals and treatment regimens for rabies. Individuals who are exposed to the rabies virus often see traditional healers for the diagnosis and treatments of the disease [7]. In different parts of Ethiopia, many traditional folk drugs were reported which were used for the treatment of rabies in both humans and animals. The most commonly cited medicinal plant species utilized for the management of rabies in Ethiopia were P*hytolacca dodecandra, Justicia schimperiana, Ricinus communis, Brucea antidysenterica, Croton macrostachyus*, and *Cucumis ficifolius* [8]. The effectiveness and safety of these traditionally used antirabies folk drugs in the country were not well demonstrated and understood. Therefore, evaluation of the antirabies activity of such medicinal plant extracts is a necessary and highly desirable task. The present study had the objectives of assessing the phytochemical constituents, acute toxicity, and antirabies potential of extracts from the leaves of *Justicia schimperiana* and *Ricinus communis* as well as the stem bark of *Croton macrostachyus.*

Methodology

Study Setting and Design: The study was carried out at Ethiopian Public Health Institute (EPHI), a Cell culture-based antirabies vaccine production laboratory and Traditional and Modern Medicine laboratory from January 2019 to August 2019 with quantitative and descriptive methods.

Study Population: Nine extracts from three plant species were evaluated on Swiss albino mice and Vero cell lines.

Plant Material Collection and Identification: Three medicinal plants were collected from Menagesha District, Ethiopia during January 2019. These were the leaves of *Justicia schimperiana and Ricinus communis* and stem bark of *Croton macrostachyus*. The plants were identified by a renowned plant taxonomist, Mr. Melaku Wondafrash, Department of Plant Biology and Biodiversity Management, Addis Ababa University, Ethiopia. The plant materials (voucher specimen numbers for *Justicia schimperiana* 001; *Croton macrostachyus* 002 and *Ricinus communis* 003) were deposited at the National Herbarium, Department of Plant Biology and Biodiversity Management, Addis Ababa University, Ethiopia.

Processing and Extract Preparation: The plant samples were properly washed, separated from foreign material, and dried in shade for 5 days (leaves) whereas, 4 weeks for the stem bark of the plant [9]. The dried samples were differently blended into a fine powder using an electric grinder and stored in air-tight containers [10]. The powdered plant materials were subjected to sequential solvent extraction with ethanol, methanol, and water accordingly at room temperature and with agitation at 120 rpm using an orbital shaker [11]. The liquid portion was then filtered using Whatman No.1 filter paper [12]. The filtrates were concentrated to dryness at 40°C using a rotary evaporator to obtain the crude extracts while water extracts were lyophilized. Resultant dried extracts were reconstituted in a dimethyl sulfoxide ethanol mixture (60:40, v/v) and the stock solutions were sterilized by using a 0.45 µm syringe filter and stored at - 20°C before use [11].

Phytochemical Screening: The extracts were screened for the presence and absence of different phytochemical constituents to relate the secondary metabolites with antirabies activity. Tests for alkaloids, flavonoids, phenol, steroids, saponins, tannins, terpenoids were carried out following standard procedures described by [13-17]. Briefly, the test for alkaloids was carried out by subjecting a few ml of each plant extract to a few drops of Wagner's Reagents (iodine solution in potassium iodide). For flavonoids, an Alkaline reagent test was adopted; the plant extract was treated with 2-3 drops of sodium hydroxide solution and some drops of sulphuric acid. The test for phenol was carried out by subjecting 50 mg of each plant extract in 5 ml of distilled water, filtered and 5% ferric chloride solution added to the filtrate. The presence of steroids was determined by subjecting 1ml of extract in 1ml of chloroform, 2-3 ml of acetic anhydride, and 1 to 2 drops of concentrated sulphuric acid. To determine the presence of tannins, 1 to 2 drops of diluted ferric chloride solution was added to 2 ml of the aqueous extract. For the confirmation of terpenoids, 0.8 g of plant extract diluted with 5 ml of methanol were subjected to 2 ml of chloroform and 3 ml of sulphuric acid. The extracts were subjected to a frothing test for the identification of saponins.

Cell Culture and Virus

The African green monkey kidney (Vero) cell line was obtained from Vaccine and Diagnostics production directorate, EPHI, Ethiopia. The cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) (Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10 % fetal bovine serum (FBS), 10,000 units' penicillin, and 10 mg streptomycin/mL (Sigma-Aldrich, St. Louis, MO, USA), and 3.7 g/L sodium bicarbonate at pH 7.4. Cells were maintained at 37°C under a humidified 5 % CO_2 atmosphere. Passage number 36–42 was maintained for bioassay.

The Department of Vaccine and Diagnostics production directorate, Ethiopian public health Institute, provided rabies virus Pasteur virus strain (RV PV) used in the present study. The virus (PV) was propagated in Vero cells as previously described by and the titer of infectious virus was obtained by the limit-dilution method and expressed as 50% tissue culture infections dose per ml (TCID50/ml) [18]. Briefly, serial 10-fold dilutions (10-1 to 10-7) of virus in serum-free MEM were added into the confluent monolayer in 96-well tissue culture plate and were incubated for 72 h at 37°C. After incubation, the medium was decanted and the cells were fixed by adding cold acetone (50 µl per well) and kept at 4°C for 30 minutes. After discarding the acetone, cells were stained by the direct polyclonal fluorescent-labeled antibody for 30 minutes and washed three times with 0.01 M phosphate buffer saline (PBS), air

dried, and visualized under an inverted fluorescence microscope. The titer was calculated by using the Spearman-Karber method and expressed as TCID50. The virus titration was also performed in mice through intracerebral inoculation using serial ten-fold dilutions at a volume of 0.03 ml. Six mice were inoculated per virus dilution, and endpointoint was death. The statistical method of Spearman-Karber was used and the mortality at each dilution was calculated to determine the 50% endpoint titer [19].

Animals and Acute Toxicity Tests

Female Swiss albino mice aged 3-4 weeks and with the weight of 15-25gobtained from the laboratory animal unit of Ethiopian Public Health Institute (EPHI) were used for the studies. The acute toxicity studies were conducted according to OECD (Organization for Economic Cooperation and Development) Guideline 425. After 12 h of fasting, the animals were divided into five groups. The treatments were performed by single oral administration as doses of 1000, 2000, 3000, 4000 and 5000 mg/kg of body weight. The animals were observed for signs of toxicity over 14 days. For the determination of LD50 of extracts, an exploratory assay with each tested mouse strain was performed to exactly determine the dose range to be used with accuracy. All observations were systematically recorded, with individual records being maintained for each mouse and at the end of the test surviving animals were humanely killed by cervical dislocation [20].

In Vivo Antirabies Assay

The in vivo antirabies activities of plant extracts at the dose of 3000 mg/kg were compared with negative control based on the difference in mean survival time of a group of mice challenged with rabies virus (PV). As described by Asefa, the test group of Swiss albino mice (6 mice/cage) was inoculated intracerebrally (30 µl) with the challenge dose of 10 LD50 RV PV followed by the oral administration (after 6 h) of the extracts diluted with 4% Polysorbate-80 solvent by using an intra-gastric needle based on the animal's body weight [21]. The volume administered was calculated based on individual mouse body weight and 0.5 ml was the maximum volume administered.

Determination of Mean Survival Time

Anti-rabies activities of the selected medicinal plant extracts were evaluated on mice survival period compared to the negative control group. Mortality rates as a result of the rabies virus challenge were determined by clinical signs and a direct fluorescent antibody test [22]. The mice were monitored daily for sign of paralysis and mortality for about 28 days after inoculation of the virus. Death was recorded for each mouse in the treatment and control groups throughout the follow-up period on the mouse history cards. The number of days each mouse survived was recorded for the mice in each group and mean survival times were calculated using the formula [23].

$$MST = \frac{\text{Sum of days of survival of mice/group}}{\text{total number of mice in the group}}$$

Confirmatory diagnosis of rabies through direct Fluorescent antibody test (FAT) was conducted by the opening of the skulls and collection of the brain of mice was done according to the procedure specified by [24]. Brain impressions were made upon microscope slides, which were fixed by acetone and incubated with fluorescein isothiocyanate (FITC)-labeled antibodies to the rabies virus. The stained impressions were viewed using fluorescence microscopy [25]. When the labeled antibody is incubated with rabies suspect brain tissue, it will bind to rabies antigen [26].

In-Vitro Cytotoxicity Test

An MTT assay previously described by Chan with some modifications was followed to evaluate the toxicity of the *leaves of Justicia schimperiana* and *Ricinus communis* and stem bark of Croton macrostachyus on Vero cells (from African green monkey kidney cells) [11]. Briefly, a confluent Vero cell monolayer with the cell density of 4 × 104 cells/well were seeded in 96-well flat-bottom microtiter plate and allowed to adhere for 24 h at 37°C in a CO_2 incubator. One hundred (100 µl) of each two-fold serially diluted extract (concentrations ranging from12.8 mg/ml to 0.4 mg/ml) in DMEM was added to the wells. Medium control and cell control (cells without extract treatment) were also incorporated in the same plates. The plates were incubated for another 72 h at 37 °C in a 5 % CO_2 incubator. A 20 µl of MTT solution (5 mg/ml in phosphate buffer solution) was pipetted into each well followed by a 3-hour incubation period at 37°C in the 5 % CO_2 incubator. After incubation, MTT was aspirated, and the formed formazan crystals were solubilized by adding 50µl of DMSO per well, followed by gentle shaking for 15 min [27]. The intensity of the dissolved formazan crystals (purple color) was quantified using the ELISA plate reader at 570 nm. All the experiments were performed in triplicate.

Invitro Antirabies Assay

The in vitro antirabies activity of the plant extracts was evaluated by FAT assay. Vero cell lines were trypsinized and 4 × 104 cells per well were seeded in a 96-well tissue culture microplate and incubated at 37°C for 72 h. After the incubation period, 50µl of rabies virus suspension was added to confluent cell monolayers in a 96-well plate and allowed to stand for 1h to enable virus adsorption. Thereafter, different concentrations (2mg/ml, 4mg/ml, and 8mg/ml) of each extract based on cytotoxicity test result were added in triplicate into all the wells except the negative control wells that contained only Vero cells and the virus control that contained an equal virus concentration but lacked the plant extract. The plates were incubated at 37°C in a 5% CO_2 humidified incubator for 72 h. The medium was decanted and the cells were fixed by adding cold acetone (50µl per well) and kept at 4°C for 30 minutes. After discarding the acetone, cells were stained by the direct polyclonal fluorescent-labeled antibody for 30 minutes and then washed (3 times) with 0.01 M PBS, air dried, and visualized under an invert-

ed fluorescence microscope. Reading was qualitative, every well that shows specific fluorescence was considered to be positive.

Data Analysis

Data were entered into an excel spreadsheet and then transferred to a statistical package for social sciences (SPSS version 20). The student's t-test was used to compare means of treatment and control groups and to evaluate the significance of observed differences between groups of mice in the mean survival time (Days). The cell viability was calculated as [(x - y)/ (z - y)] × 100 %, where x, y, and z were average absorbance of cells treated with extract, average absorbance of medium control, and average absorbance of cell control, respectively. The 50% cytotoxic (CC50) and 50% effective (EC50) concentrations were calculated from concentration-effect curves after linear the 50% cytotoxic (CC50) and 50% effective (EC50) concentrations were calculated from concentration-effect curves after linear regression analysis.

Results and Discussion

Phytochemical Study Result of Extracts

The successive extracts of leaves of *Justicia schimperiana* and *Ricinus communis* and stem bark of *Croton macrostachyus* have revealed the presence of alkaloids, flavonoids, steroids, phenols, tannins, and terpenoids and the absence of saponins (Table 1). All extracts from the three plants showed positive results for alkaloids, but negative for saponins. From the three plants studied, only *Croton macrostachyus* showed flavonoids in its three extracts. The Libermann Burchard test failed to show the presence of steroids in the ethanol extract but gave a positive result with the methanolic and water extracts. Thus, the preliminary screening tests may be useful in the detection of the bioactive principles and subsequently may lead to drug discovery and development.

Table 1: Different phytochemical components in the ethanol, methanol and, water extracts of the leaves of Justicia schimperiana and Ricinus communis and the stem bark of Croton macrostachyus plants

Plant name	Solvent	Secondary metabolites						
		Alkaloids	Flavonoids	Phenols	Steroids	Saponins	Tannins	Terpenoids
Justicia schimperiana	Ethanol	+	_	_	+	_	+	_
	Methanol	+	+	+	+	_	+	+
	Water	+	+	_	_	_	_	+
Croton macrostachyus	Ethanol	+	+	+	_	_	+	+
	Methanol	+	+	+	+	_	+	+
	Water	+	+	_	+	_	_	+
Ricinus communis	Ethanol	+	_	+	+	_	+	+
	Methanol	+	+	+	+	_	+	+
	Water	+	_	+	+	_	+	+
Where, + =Presence and _ = Absence								

The Result of Acute Oral Toxicity Test

The acute toxicity results showed no evidence of the toxicity of the ethanol, methanol, and water extracts of Justicia schimperiana (leaf), *Croton macrostachyus* (stem bark), and Ricinus communis (leaf) in mice administered at 1000 mg/kg, 2000 mg/kg and 3000 mg/kg. No abnormalities were recorded at three doses about food consumption, water intake, and bodyweight of the mice. This shows that the plant extracts could be well tolerated up to the dose of 3000 mg/kg body weight of Swiss albino mice. However, at higher doses, i.e. 4000 mg/kg and 5000 mg/kg, mice showed common signs of toxicity like low locomotion, weakness, and erection of hairs including death in the course of acute study. As a result, the LD50 of the extracts could be greater than 3000mg/kg body weight.

The log dose at probit 5.0 (Log LD50) for the ethanol, methanol, and aqueous extracts of *Justicia schimperiana* were found to be 3.60, 3.54, and 3.6,5, and hence, LD50 was calculated by taking the antilog of the Log LD50 values of each extract and found to be 4000mg/kg,3500mg/kg and 4500mg/kg body weight respectively. The LD50 for the ethanol and methanol extracts of *Croton macrostachyus* was found to be 3500mg/kg body weight. Whereas, the aqueous extract was found to be 3900mg/kg. The LD50 values for the ethanol and aqueous extracts of *Ricinus communis* were found to be 3500mg/kg but 4000 mg/kg for the methanol extract of the plant. It suggested that the extracts may not be completely safe at a dose higher than 3000 mg/kg. According to the toxicity scale, the ethanol, methanol, and aqueous extracts of *Justicia schimperiana* (leaf), *Croton macrostachyus* (stem bark), and *Ricinus communis* (leaf) were placed in category IV (500 mg/kg-5000 mg/kg, p.o.), and hence classified as slightly toxic [28].

The log dose at probit 5.0 for the methanol extracts of J*usticia schimperiana, Croton macrostachyus*, and *Ricinus communis* were found to be 3.54, 3.54, and 3.60 respectively (Figure 1). A graph was plotted between probit vs. Log dose and LD50 values of extracts were confirmed by the graph which was the dose at probit 5.0, i.e. 50% mortality.

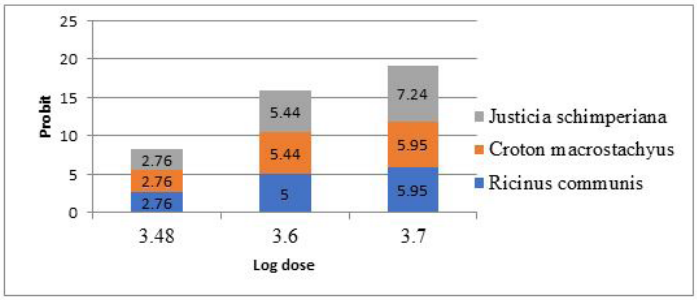

Figure 1: LD50 Values of Methanol Extracts from the Three Plant Extracts

In Vivo Antirabies Potential of Plant Extracts

A group of mice infected with rabies virus but not treated with any of plant extracts showed a 0% survival rate and a mean survival period of 9.5 days. However, oral treatment of mice infected with rabies virus with ethanol, methanol, and water extracts of the leaves of *Justicia schimperiana* and *Ricinus communis* as well as the stem bark of Croton macrostachyus at a dose of 3000 mg/kg significantly ($p<0.05$) increased the mean survival time compared to those of negative control group. Relatively higher mean survival time was obtained when methanol extract of *Ricinus communis* was administered to mice at a dose of 3000 mg/kg. The mean survival time of mice treated with methanol extracts of the leaves of *Justicia schimperiana*, the stem bark of Croton macrostachyus, and the leaves of Ricinus communis were 14.3, 21.3, and 22.16 days, respectively (Figure 2).

One mouse from the negative control group died within four days of inoculation with PV but, didn't show any antigen against rabies virus which indicates the death was due to accidental rather than the effect of the virus. Death from samples taken from those at moribund state was due to the action of rabies virus PV strain because all the samples showed a viral antigen but, most of the samples from survivors indicate negative for antigen detection.

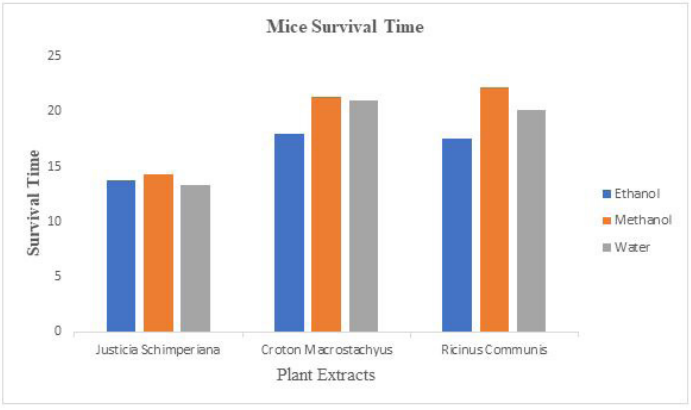

Figure 2: Antirabies Effect of Plant Extracts on Mice Survival Time (n=6) at 3000mg/kg

Cytotoxicity Determination of Plant Extracts in Vero Cell Line

Evaluation of extracts on Vero cell lines by using MTT assay showed that the 50% cytotoxic concentration (CC50) values of the ethanol and aqueous extracts of three plants; *Justicia schimperiana, Croton macrostachyus*, and *Ricinus communis* were found to be above 12.8 mg/ml. However, the 50% cytotoxic concentration (CC50) value of the methanol extracts from *Justicia schimperiana* and *Croton macrostachyus* was found to be 9.6mg/ml and 8mg/ml whereas, *Ricinus communis* showed below12.8 mg/ml (Figure 3). Generally, the percentage viability was found to be increasing with decreasing concentration of test extracts. The MTT assay results revealed that all extracts tested were non-cytotoxic and exhibited CC50 values above the cut-off point which is 30µg/ml. Extracts can be considered non-cytotoxic if the CC50 is higher than 30µg/ml [29].

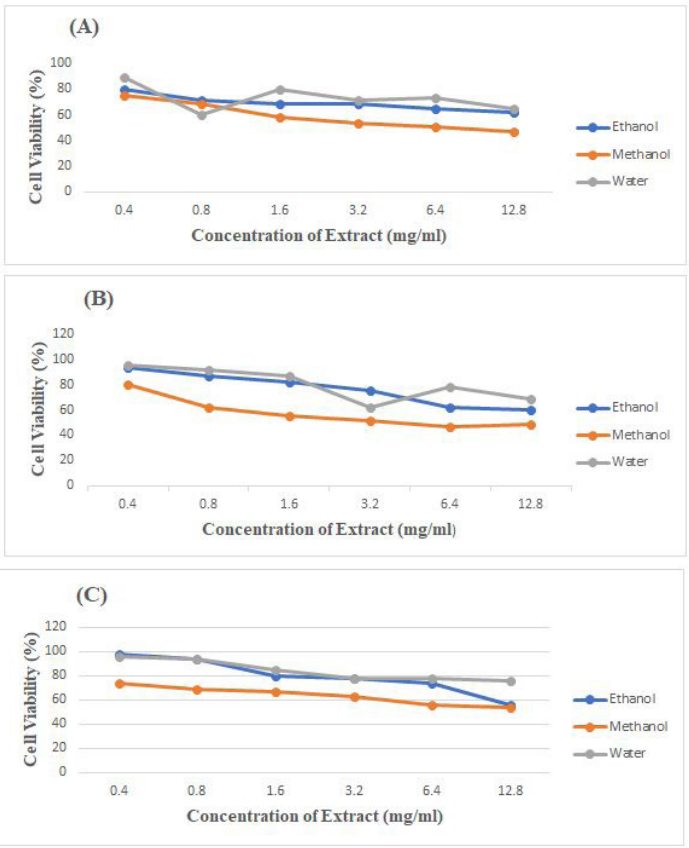

Figure 3: Effect of 9 Extracts of Three Medicinal Plants on the Viability of Vero Cells Based on MTT assay. (A)– *Justicia schimperiana*; (B)– *Croton macrostachyus*; (C)– *Ricinus communis*

In Vitro Antirabies Effect of Plant Extracts

The in vitro antirabies activity of medicinal plant extracts was evaluated by fluorescent antibody assay then, the IC50 value and the selective index (SI) were calculated (Table 2). Confluent monolayers of Vero cells on 96 well microtiter plates, which were infected with rabies PV strain (A), and rabies-infected Vero cells which were treated with different extracts (B) were compared (Figure 4).

Although, differences were observed between antirabies activities of the extracts, each of the extracts tested in the present study displayed antirabies activity against PV strain.

Methanol extracts from all plant samples at a concentration of 8mg/ml showed a 100% RV-PV inhibition. The half-maximal inhibitory concentration (IC_{50}) values ranged from 3mg/ml to 6mg/ml with the selectivity indices (SI) of above 2.13 for each tested material. The water extracts of all tested samples and the ethanol extract of *Justicia schimperiana* showed relatively the highest IC50 values. The results exhibit that the smaller the IC_{50} value higher the antirabies activity. Ethanol extracts of *Croton macrostachyus* stem bark and *Ricinus communis* leaf as well as methanol extract of *Ricinus communis* show the highest SI value against RV-PV with the value of >4.27. The selectivity indices ($SI = CC_{50}/IC_{50}$) of *Ricinus communis* (leaf) methanol extract (SI>4.27) was found to be higher than the methanol extracts of *Justicia schimperiana* (SI=3.2) and *Croton macrostachyus* (SI=2.7). SI values are less than one considered as weak, greater than one moderate, and greater than three considered as good antiviral activities (Bagla et al. 2012). The IC_{50} value and the selective index(SI) of ethanol, methanol, and aqueous extracts of *Justicia schimperiana* (leaf) were calculated and found to be 6mg/ml (SI>2.13), 3mg/ml (SI=3.2), and 6mg/ml (SI>2.13) respectively. The methanol extract exhibited a smaller IC_{50} value when compared to the other extracts. *Croton macrostachyus* stem bark showed 50% inhibition of RV-PV at 3mg/ml (ethanol and methanol extracts) and 6mg/ml (aqueous extracts). The ethanol extract of *Croton macrostachyus* stem bark contains antirabies active compounds with good activity (SI>4.27) whereas, the methanol (SI=2.7) and aqueous (SI>2.13) extracts had shown moderate antirabies activity against PV strain. The IC_{50} values of ethanol, methanol and aqueous extracts of Ricinus communis (leaf) was found to be 3 mg/ml (SI> 4.27), 3mg/ml (SI> 4.27) and 6 mg/ml (SI>2.13) respectively. This indicates that methanol and ethanol extracts had good antirabies activity against PV strain while aqueous extract with moderate activity.

Table 2: Cytotoxicity (CC50), inhibition concentration (IC50), and selectivity index (SI) of plant extracts

Plant name	Solvent	CC50	IC50	SI
Justicia schimperiana	Ethanol	>12.8 mg/ml	6 mg/ml	> 2.13
	Methanol	9.6 mg/ml	3 mg/ml	3.2
	Aqueous	>12.8 mg/ml	6 mg/ml	> 2.13
Croton macrostachyus	Ethanol	>12.8 mg/ml	3 mg/ml	> 4.27
	Methanol	8 mg/ml	3 mg/ml	2.7
	Aqueous	>12.8 mg/ml	6 mg/ml	> 2.13
Ricinus communis	Ethanol	>12.8 mg/ml	3 mg/ml	> 4.27
	Methanol	>12.8 mg/ml	3 mg/ml	> 4.27
	Aqueous	>12.8 mg/ml	6 mg/ml	> 2.13

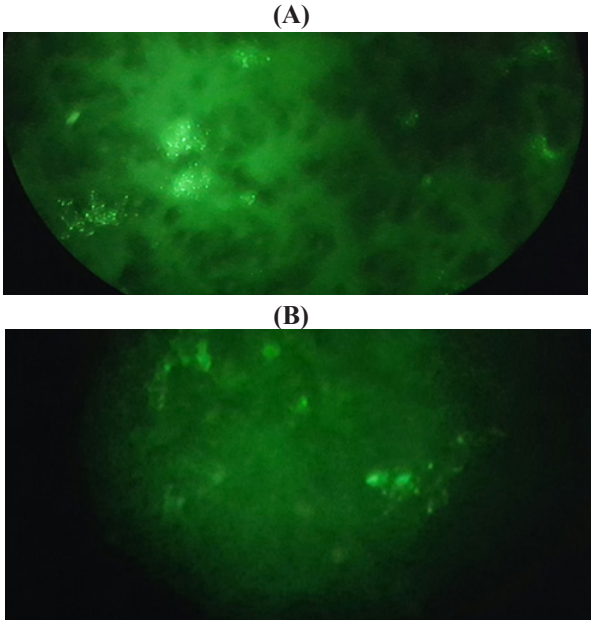

Figure 4: Microscopic photographs of Vero cell lines infected with rabies virus (A) and rabies-infected Vero cells which were treated with different extracts (B).

Cells were observed with a fluorescence microscope.

Conclusion

The phytochemical analysis showed that the ethanol, methanol, and water extract of the leaves of *Justicia schimperiana* and *Ricinus communis* as well as the stem bark *Croton macrostachyus* contain a mixture of phytochemicals as alkaloids, flavonoids, phenols, steroids, tannins, and terpenoids but lack saponins. All the extracts were slightly toxic in the animal model but noncytotoxic in Vero cell lines. All the Plant extracts had a moderate to good antirabies activity against PV strain. The methanol plant extracts gave more antirabies activity compared to ethanol and water extracts in the mice model [30-45].

List of abbreviations

BHK Baby hamster kidney
CC50 Cytotoxicity Concentration 50%
CM Croton macrostachyus
IC50 Inhibitory Concentration 50%
JS Justicia schimperiana
LD50 Medium Lethal Dose
MTT 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

PEP	Post-Exposure Prophylaxis
PrEP	Pre-Exposure Prophylaxis
PV	Pasteur Virus
RC	Ricinus communis

Declarations

Ethics Approval and Consent to Participate

Ethical approval for the study was sought from the College of Natural & Computational Science Institutional Review Board (CNS IRB), Addis Ababa University, Ethiopia. The animals were handled according to the international guidelines for the care and use of laboratory animals. For the care and use of animals, the ARRIVE guidelines (Animal Research: Reporting of in Vivo Experiments) were followed [46].

This study was carried out in strict accordance with the recommendations in the National Environmental Management: Biodiversity Act 101 of 2004, the IUCN Policy Statement on Research Involving Species at Risk of Extinction, and Ethiopian Environment and Forest Research Institute Establishment Council of Ministers Regulation (Regulation No. 327/2014). No approvals were required for the study, which complied with all relevant regulations. The botanical identification of plant species was carried out by a renowned plant taxonomist, Mr. Melaku Wondafrash, Department of Plant Biology and Biodiversity Management, Addis Ababa University, Ethiopia. The plant materials (voucher specimen numbers for Justicia schimperiana 001; Croton macrostachyus 002 and Ricinus communis 003) were deposited at the National Herbarium, Department of Plant Biology and Biodiversity Management, Addis Ababa University, Ethiopia.

Consent to Participate

Not applicable

Consent for Publication

Not applicable

Availability of Data and Materials

All data generated or analyzed during this study are included in this manuscript. Data presented are mainly results obtained from the study and have not been submitted or published elsewhere for publication. The datasets (Plant samples) supporting the conclusions of this article are available in the National Herbarium, Department of Plant Biology and Biodiversity Management, Addis Ababa University, Ethiopia.

Competing Interests

Authors declare that they need no competing interests.

Funding

The authors received no financial support for the research, authorship, and/or publication of this article.

Authors' Contributions

All authors contributed to and approved the manuscript. Yeweynshet Tesera and Asnake Desalegn conceived and planned the presented experiments. Birhanu Hurisa and Anberbir Alemu contributed to sample preparation. Jemal Mohammed and Sileshi Degu carried out the experiments. Yeweynshet Tesera wrote the manuscript with support from Ashenif Tadele, Ayele Bizuneh, and Abebe Mengesha. Demise Mulugeta and Sintayehu Ashenafi contributed to the analysis and/or interpretation of the results. All authors provided critical feedback and revised the manuscript critically for important intellectual content.

Acknowledgments

We would like to acknowledge Mr. Eyob Debebe and Mr. Asfaw Meresa for their sincere technical supports. We would also acknowledge the Ethiopian Public Health Institute and Addis Ababa University for helping us to successfully complete a Master of Science degree (MSc) in Applied Microbiology.

References

1. Zulu, G. C., Sabeta, C. T., & Nel, L. H. (2009). Molecular epidemiology of rabies: focus on domestic dogs (Canis familiaris) and black-backed jackals (Canis mesomelas) from northern South Africa. Virus Research, 140(1-2), 71-78.
2. Vural, S. A., Bozkurt, M. F., Özkara, A., Alçığır, M. E., & İlhan, F. S. (2016). Apoptosis in natural rabies virus infection in dogs. J Vet Res 60(3): 227-231.
3. Jackson, A. C., Warrell, M. J., Rupprecht, C. E., Ertl, H. C., Dietzschold, B., O'reilly, M., ... & Wilde, H. (2003). Management of rabies in humans. Clinical Infectious Diseases, 36(1), 60-63.
4. Knobel, D. L., Cleaveland, S., Coleman, P. G., Fèvre, E. M., Meltzer, M. I., Miranda, M. E. G., ... & Meslin, F. X. (2005). Re-evaluating the burden of rabies in Africa and Asia. Bulletin of the World health Organization, 83, 360-368.
5. FEKADU, M. (1982). Rabies in Ethiopia. American journal of epidemiology, 115(2), 266-273.
6. Reta, T., Teshale, S., Deresa, A., Ali, A., Mengistu, F., Sifer, D., & Freuling, C. M. (2014). Rabies in animals and humans in and around Addis Ababa, the capital city of Ethiopia: A retrospective and questionnaire based study. Journal of veterinary medicine and animal health, 6(6), 178-186.
7. Oyda, S., & Megersa, B. (2017). A review of rabies in livestock and humans in ethiopia. International Journal of Research-Granthaalayah, 5(6), 561-577.
8. Meresa, A., Degu, S., Tadele, A., Geleta, B., Moges, H., Teka, F., & Fekadu, N. (2017). Medicinal plants used for the management of rabies in Ethiopia–a review. Med Chem (Los Angeles), 7, 795-806.
9. Doughari, J. H., & Manzara, S. (2008). In vitro antibacterial activity of crude leaf extracts of Mangifera indica Linn. Afr J Microbiol Res, 2(4), 67-72.
10. Hussain, I., Ullah, R., Khurram, M., Ullah, N., Baseer, A.,

Khan, F. A., ... & Khan, N. (2011). Phytochemical analysis of selected medicinal plants. African Journal of Biotechnology, 10(38), 7487-7492.
11. Chan, S. M., Khoo, K. S., & Sit, N. W. (2015). Interactions between plant extracts and cell viability indicators during cytotoxicity testing: implications for ethnopharmacological studies. Tropical Journal of Pharmaceutical Research, 14(11), 1991-1998.
12. Wagura, A. G., Wangai, S. O., Manguro, L., & Gichimu, B. M. (2011). Effects of Selected Plants' Extracts on in vitro Growth of Rahtonia slanacearum (Smith), the Causal Agent of Bacterial Wilt of Irish Potatoes. Plant Pathology Journal, 10, 66-72.
13. Banu, K. S., & Cathrine, L. (2015). General techniques involved in phytochemical analysis. International Journal of Advanced Research in Chemical Science, 2(4), 25-32.
14. Khalid, S., Shahzad, A., Basharat, N., Abubakar, M., & Anwar, P. (2018). Phytochemical screening and analysis of selected medicinal plants in Gujrat. Journal of Phytochemistry and Biochemistry, 2(1), 1-3.
15. Sasidharan, S., Chen, Y., Saravanan, D., Sundram, K. M., & Latha, L. Y. (2011). Extraction, isolation and characterization of bioactive compounds from plants' extracts. African journal of traditional, complementary and alternative medicines, 8(1), 1-10.
16. Wadood, A., Ghufran, M., Jamal, S. B., Naeem, M., Khan, A., & Ghaffar, R. (2013). Phytochemical analysis of medicinal plants occurring in local area of Mardan. Biochem Anal Biochem, 2(4), 1-4.
17. Zohra, S. F., Meriem, B., Samira, S., & Muneer, M. A. (2012). Phytochemical screening and identification of some compounds from mallow. J Nat Prod Plant Resour, 2(4), 512-516.
18. Webster, L. T., & Clow, A. D. (1937). Propagation of rabies virus in tissue culture. The Journal of Experimental Medicine, 66(1), 125-131.
19. Ramakrishnan, M. A. (2016). Determination of 50% endpoint titer using a simple formula. World journal of virology, 5(2), 85-86.
20. OECD, O. (2001). 423-Guidelines for the Testing of Chemicals Acute Oral Toxicity-Fixed Dose Procedure. Animals.
21. Deressa, A., Hussen, K., Abebe, D., & Gera, D. (2010). Evaluation of the Efficacy of Crude Extracts of Salix subserrata and Silene macroselen for the treatment of rabies in Ethiopia. Ethiopian Veterinary Journal, 14(2), 1-16.
22. Admasu, P., Deressa, A., Mengistu, A., Gebrewold, G., & Feyera, T. (2014). In vivo antirabies activity evaluation of hydroethanolic extract of roots and leaves of Phytolacca dodecandra. Glob Vet, 12(1), 12-18.
23. Nafiu, M. O., Abdulsalam, T. A., & Akanji, M. A. (2013). Phytochemical analysis and antimalarial activity aqueous extract of Lecaniodiscus cupanioides root. Journal of tropical medicine, 14, 1-4.
24. Dean DJ, MK Abelseth. (1973). The fluorescent antibody test. Monogr Ser World Health Organ (23), 73-84.
25. Mayes, B., & Rupprecht, C. E. (2015). Direct fluorescent antibody test for rabies diagnosis. In Current Laboratory Techniques in Rabies Diagnosis, Research and Prevention, Volume 2 (pp. 83-92). Academic Press.
26. Hosseini, P. and Asgary, V. (2015). Postmortem Diagnosis of Rabies in Animal Brain by Fluorescent Antibody Testing. Intl. Conf. on AABES. 146-147.
27. Fayyad, A. G., Ibrahim, N., & Yaakob, A. W. (2014). Phytochemical screening and antiviral activity of Marrubium vulgare. Malays J Microbiol, 10(2), 106-111.
28. Hodge, H. C., & Sterner, J. H. (1943). Determination of substance acute toxicity by LD50. Am Ind Hyg Assoc, 10, 93-96.
29. Nondo, R. S., Moshi, M. J., Erasto, P., Zofou, D., Njouendou, A. J., Wanji, S., ... & Titanji, V. P. (2015). Evaluation of the cytotoxic activity of extracts from medicinal plants used for the treatment of malaria in Kagera and Lindi regions, Tanzania. Journal of Applied Pharmaceutical Science, 5(4), 007-012.
30. Admasu, P., Mekonnen, Y., & Wold, G. G. (2014). Rabies and its folk drugs remedies in Ethiopia: a review. International Journal of Basic and Applied Virology, 3(2), 22-27.
31. Ammerman, N. C., Beier-Sexton, M., & Azad, A. F. (2008). Growth and maintenance of Vero cell lines. Current protocols in microbiology, 11(1), A-4E.
32. Chernet, B., & Nejash, A. (2016). Review of Rabies preventions and control. Int. J. Life Sci, 4(2), 293-301.
33. Hurisa, B., Mengesha, A., Newayesilassie, B., Kerga, S., Kebede, G., Bankovisky, D., ... & Urga, K. (2013). Production of cell culture based anti-rabies vaccine in Ethiopia. Procedia in Vaccinology, 7, 2-7.
34. Blanton, J. D., Palmer, D., & Rupprecht, C. E. (2010). Rabies surveillance in the United States during 2009. Journal of the American Veterinary Medical Association, 237(6), 646-657.
35. Crowcroft, N. S., & Thampi, N. (2015). The prevention and management of rabies. Bmj, 350, 1-9.
36. Hemachudha, T., Ugolini, G., Wacharapluesadee, S., Sungkarat, W., Shuangshoti, S., & Laothamatas, J. (2013). Human rabies: neuropathogenesis, diagnosis, and management. The Lancet Neurology, 12(5), 498-513.
37. Okonko, I. O., Adedeji, O. B., Babalola, E. T., Fajobi, E. A., Fowotade, A., & Adewale, O. G. (2010). Why is there still rabies in the world?-an emerging microbial and global health threat. Global Veterinarian, 4(1), 34-50.
38. Abdela, J., Engidawork, E., & Shibeshi, W. (2014). In vivo antimalarial activity of solvent fractions of the leaves of justicia schimperiana hochst. Ex Nees against Plasmodium berghei in Mice. Ethiopian Pharmaceutical Journal, 30(2), 95-108.
39. Jibat, T., Mourits, M. C., & Hogeveen, H. (2016). Incidence and economic impact of rabies in the cattle population of Ethiopia. Preventive Veterinary Medicine, 130, 67-76.
40. Kaare, M., Lembo, T., Hampson, K., Ernest, E., Estes, A., Mentzel, C., & Cleaveland, S. (2009). Rabies control in rural Africa: evaluating strategies for effective domestic dog vaccination. Vaccine, 27(1), 152-160.
41. Kuzmin, I. V., & Rupprecht, C. E. (2008). Rabies Virus. Ency-

clopedia of Virology, 367-373.
42. Leung, A. K., Davies, H., & Hon, K. L. E. (2007). Rabies: epidemiology, pathogenesis, and prophylaxis. Advances in therapy, 24(6), 1340-1347.
43. Nie, J., Wu, X., Ma, J., Cao, S., Huang, W., Liu, Q., ... & Wang, Y. (2017). Development of in vitro and in vivo rabies virus neutralization assays based on a high-titer pseudovirus system. Scientific reports, 7(1), 1-12.
44. Singh, R., Singh, K. P., Cherian, S., Saminathan, M., Kapoor, S., Manjunatha Reddy, G. B., ... & Dhama, K. (2017). Rabies–epidemiology, pathogenesis, public health concerns and advances in diagnosis and control: a comprehensive review. Veterinary Quarterly, 37(1), 212-251.
45. Soltanian, S., Sheikhbahaei, M., & Mohamadi, N. (2017). Cytotoxicity evaluation of methanol extracts of some medicinal plants on P19 embryonal carcinoma cells. J Appl Pharm Sci, 7(7), 142-149.
46. Percie du Sert, N., Hurst, V., Ahluwalia, A., Alam, S., Avey, M. T., Baker, M., ... & Würbel, H. (2020). The ARRIVE guidelines 2.0: Updated guidelines for reporting animal research. Journal of Cerebral Blood Flow & Metabolism, 40(9), 1769-1777.